PRELIMINARY STUDY OF PUFA PRODUCTION BY MICROALGAE AND CYANOBACTERIA USING TWO-PHASE SYSTEM

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Abstract. Docosahexaenoic acid (DHA) and gamma-linolenic acid (GLA) are widely used as valuable polyunsaturated fatty acids for uses in the pharmaceutical, cosmetic and food industries as therapeutical and functional ingredient with added beneficial effects in human health.

The marine heterotrophic microalga *Cryptecodinium cohnii* and the freshwater autotrophic cyanobacterium *Arthrospira (Spirulina) maxima* are promising DHA and GLA sources, respectively, chosen as living models to be grown in fermenters and flat alveolar photobioreactors, respectively. Most microorganisms require molecular oxygen for the desaturation mechanism in the biosynthesis pathways of long polyunsaturated fatty acids, namely GLA and DHA., so availability of oxygen generally determines the degree of unsaturation of microbial fatty acids. On the other hand inefficient oxygen removal under autotrophic growth, mainly under high light intensities has a detrimental effect on biomass productivity as undesired phenomena (photoinhibition and photooxidation) could occur. Four water immiscible biocompatible solvents (log P≥6.6) showing high oxygen solubility were added to shaken flasks cultivations of *Arthrospira (Spirulina) maxima* and *Cryptecodinium cohnii* order to act as oxygen carriers: dodecane, hexadecane, di-(2-ethylhexyl)phthalate-BEHP and Fluorinert® FC-70 with or without a surfactant (-sodium dodecylsulphate-SDS).

The addition of BEHP to *Arthrospira (Spirulina) maxima* cultures increased the GLA, content reaching 0.009 g GLA (g biomass^{-1}), at the solvent concentration of 0.25 % (v/v). FC-70 also increased the GLA content (0.009 g GLA. (g biomass^{-1})at 0.1 % (v/v). The presence of BEHP increased the DHA percentage, attaining about 40% of the total fatty acids at the organic solvent concentration of 0.1% (v/v).

1. Introduction

Owing to the medical and nutritional potential of polyunsaturated fatty acids (PUFA), there has been considerable interest in their production. Gamma-linolenic acid (GLA; omega-6 fatty acid), an essential polyunsaturated fatty acid in the form of evening primrose oil has been used to treat rheumatoid arthritis, multiple sclerosis, schizophrenia, atopic eczema and premenstrual syndromes. Docosahexaenoic acid (DHA, omega-3 fatty acid) has physiological effects in main three areas, i.e., heart and circulatory, inflammatory and cancer. Recently, there has been considerable interest in microbial production of PUFA which is now considered an economical alternative way to produce large quantities of fatty acids (Kennedy et al, 1993).

The autotrophic cyanobacterium *Arthrospira (Spirulina) maxima* has been referred as an important source of GLA (Reis et al., 1998; Mendes et al., 2005). The heterotrophic microalga *Crypthecodinium cohnii* is an interesting source for DHA production (Kyle, 1996; Kyle et al., 1992) due to its unique fatty acid

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composition. *C. cohnii* can accumulate relatively high amounts of lipid (20%) with 30–50% DHA of the fatty acids and no other polyunsaturated fatty acids present above 1% (Harrington and Holz, 1968; Beach and Holz, 1973).

Inefficient oxygen removal under autotrophic growth, mainly under high light intensities may have a detrimental effect on biomass productivity as undesired phenomena (photoinhibition and photooxidation) could occur (Molina et al., 2001). However, microorganisms require molecular oxygen for the desaturation mechanism in the biosynthesis pathways of polyunsaturated fatty acids, namely GLA and DHA, so availability of oxygen generally determines the degree of unsaturation of microbial fatty acids (Bajpai and Bajpai, 1993). Therefore, much work to improve the k_La has been done. Some limited work has been performed to observe the effect of the presence of a second organic liquid phase on oxygen mass transfer for organic compounds such as n-hexadecane (Zhao et al, 1999), n-dodecane (Hassan and Robisson, 1977) and vegetable oils (Zhao et al, 1999). By adding an organic phase with a higher affinity for oxygen, larger amounts of oxygen are removed from the aeration gas stream, being retained in the two-phase system. Whereas no more than the saturation concentration of oxygen can be dissolved in the aqueous phase, the supply of oxygen to the aqueous phase from the gas stream may be supplemented by equilibrium partitioning of dissolved oxygen from the organic phase to the aqueous phase. Beyond the oxygen availability increase in the culture, the organic solvent may contribute for lipophilic products (such as lipids) extraction and separation from the aqueous phase, preventing the product–induced feedback inhibition or toxicity and facilitating downstream product recovery (Déziel et al, 1999).

The proposed work aimed at the improvement of GLA and DHA production by the autotrophic cyanobacterium *Arthrospira (Spirulina) maxima* and the heterotrophic microalga *Crypthecodinium cohnii* respectively, through the addition of water immiscible biocompatible solvents (dodecane, hexadecane, di-(2-ethylhexyl)phthalate-BEHP and Fluorinert® FC-70 with or without a surfactant (-sodium dodecylsulphate-SDS) with a high affinity for oxygen, acting as oxygen carriers, to these microorganisms cultures.

2. Materials and Methods

2.1 Organism and Growth conditions

Arthrospira (*Spirulina*) *maxima* Geitler (LB 2342) was obtained from the University of Texas Culture Collection (Austin, USA). The microorganism was grown on the Spirulina medium (Vonshak, 1986). *Arthrosphira* biomass was harvested using a nylon plankton mesh (33μm), and then freeze-dried, grounded and stored under N_2 atmosphere at -18°C for further studies, as raw material.

Crypthecodinium cohnii was obtained from the Guillard Center for Culture of Marine Phytoplankton (CCMP), Maine, EUA. The microorganism was grown on the medium f/2 (Guillard and Ryyther, 1962; Guillard, 1975). *C. cohnii* cultures were centrifuged in a refrigerated centrifuge (Burkard Koolspin) and the pellet was freeze-dried and stored under N_2 atmosphere at -18°C for further studies.

Four organic compounds (di-ethylxiftalate (BEHP; log P= 9.6), fluoroinert (FC-70; log P= 12.9), hexadecane (log P = 8.8) and dodecane (log P = 6.6)) were added to the algae cultures, in increasing concentrations (0, 0.2, 0.25, 0.5 and 1 % (v/v) in BEHP and FC-70 0, 0.1, 0.5, 1 or 2 % (v/v) in dodecane and

hexadecane). A tensioactive agent (sodium dodecyl sulphate, SDS) was also tested by the addition of 20 µL of a 1% (p/v) SDS solution, in order to promote the dissipation of the organic phase.

A. maxima was grown in 250 mL nephlometers containing 40 mL of the growth medium, 10% (v/v) of inoculum and the organic phase in the concentrations mentioned above. The nephlometers were incubated in orbital shakers at 120 rpm, lighting by fluorescent lamps of 36 W. *C. cohnii* was also grown in nephlometers in the same conditions, but without lighting. Optical density was followed using a spectrometer Bausch & Lomb Spectronic 21, at 540 nm. Samples collected during the microorganism growth were observed under an optical microscope Olympus BH2.

2.2. Methyl Esters Preparation and Analysis

Fatty acid methyl esters were prepared by transesterification according to Lepage and Roy (1987) modified by Cohen et al. (1992). The methyl esters were then analyzed by gas-liquid chromatography, on a Varian (Palo Alto, USA) 3800 gas-liquid chromatograph (USA), equipped with a flame ionization detector. Separation was carried out on a 0.32 mm × 30 m fused silica capillary column (film 0.32 µm) Supelcowax 10 (Supelco) with helium as carrier gas at a flow rate of 1.3 ml.min^{-1}. The column temperature was programmed at an initial temperature of 200 ºC for 10 min, then increased at 4 ºC min^{-1} to 240 º C and held there for 16 min. Injector and detector temperatures were 250 and 280 ºC respectively and split ratio was 1:100. Peak identification and response factor calculation was carried out using known standards (Sigma and Nu-Chek-Prep). Heptadecanoic acid (Merck) was used as internal standard. Each sample was made in duplicate and was injected twice.

3. Results and Discussion

3.1 Growth experiments

Arthrospira (Spirulina) maxima absorbance evolution throughout the batch cultures time is shown in Figure 1. It was observed that the addition of BEHP led to an increase of the absorbance, comparing with the control experiment (0% BEHP) (Figure 1 a). This result might suggest an increase of the oxygen availability in the culture medium as a result of the addition of the organic carrier, which led an increase of the absorbance. This observation is expected as the log P value for this solvent is 9.6. According to Heipieper et al. (1994), solvents with a log P in the range 1-5 are extremely toxic, conversely to those showing a log P <1 or >5.

The presence of SDS did not increase the absorbance curves suggesting that no biomass growth enhancement occurred (Figure 1 b). Optical observations of *Arthrospira (Spirulina) maxima* cultures showed that the presence of BEHP did not changed significantly the tricome shape and size, but the addition of SDS led to the fragmentation of this microorganism filament (tricomes), in all experiments.

During the *Arthrospira (Spirulina) maxima* batch cultures evolution in the presence of FC-70, the absorbance decreased, comparing with the control experiment (0 % FC-70) (Figure 1 c) indicating this compound might be toxic for cells, although the microscopic observations did not show any cell lysis signs. The

addition of SDS seemed to have increased the absorbance of the cultures growing in the presence of FC-70, overlapping the control curve (0%

Crypthecodinium cohnii growth attained higher maximum absorbance values than *Arthrospira (Spirulina) maxima* (Figure 2). Again the presence of BEHP increased the absorbance values comparing with the control experiment SDS) (Figure 1 d). This might be due to the high affinity of the SDS for the FC-70, forming an emulsion, therefore preventing the contact of this toxic organic solvent with the cells.

Figure 1 (e) displayed the effect of hexadecane on *Arthrospira (Spirulina) maxima* growth. It was clear that the presence of this solvent led to an increase of the maximum absorbance values (maximum absorbance in the presence of hexadecane 2% (v/v), at 120 h =2.0), comparing with the previous experiments. These results might indicate that the hexadecane is non-toxic for *Arthrospira (Spirulina) maxima* cells, which was expected since its log P value is 8.8.

The effect of dodecane on *Arthrospira (Spirulina) maxima* cells is depicted in Figure 1 (f). It was clear that this solvent inhibited the growth, although no cell damage was observed under the optical microscope. (Figure 2 a). The addition of SDS also increased the absorbance relatively to the control (Figure 2 b). However, the microscopic observations revealed that, conversely to the growth in the presence of BEHP, when SDS was added a large number of cystic cells was present, indicating that the cells were growing under adverse conditions, although the absorbance increase relatively to the control .

The addition of hexadecane to *Crypthecodinium cohnii* led to an increase of the absorbance values (Figure 2 c) although the microscopic observations revealed a few cystic cells. When *Crypthecodinium cohnii* grew in the presence of dodecane, the absorbance decreased for higher solvent concentrations (0.5 and 1 %) (Figure 2 d). Moreover, the microscopic observation revealed a high number of cystic cells and, at higher solvent concentrations, cell lysis was observed.

3.2 Fatty acid extraction and quantification

Table 1 shows the *Arthrospira (Spirulina) maxima* biomass production and fatty acid composition, total fatty acids and GLA contents in biomass, in the absence/presence of BEHP and with/without the addition of SDS. It can be seen that the palmitic acid (16:0) is the major fatty acid representing about 50% of the total cellular fatty acids of this microorganism. The total fatty acids amount represents about 4 % of the biomass. The presence of BEHP increased the GLA content, reaching 0.009 g GLA.(g biomass^{-1}), at the solvent concentration of 0.25 % (v/v) (Table 1). At this solvent concentration, the total fatty acid content, as well as the GLA percentage attained their maximum values (0.062 g total fatty acids (g biomass^{-1}); 14.8 % GLA of the total fatty acids respectively). The addition of SDS decreased the GLA and the total fatty acid content, whilst the GLA percentage did not change significantly.

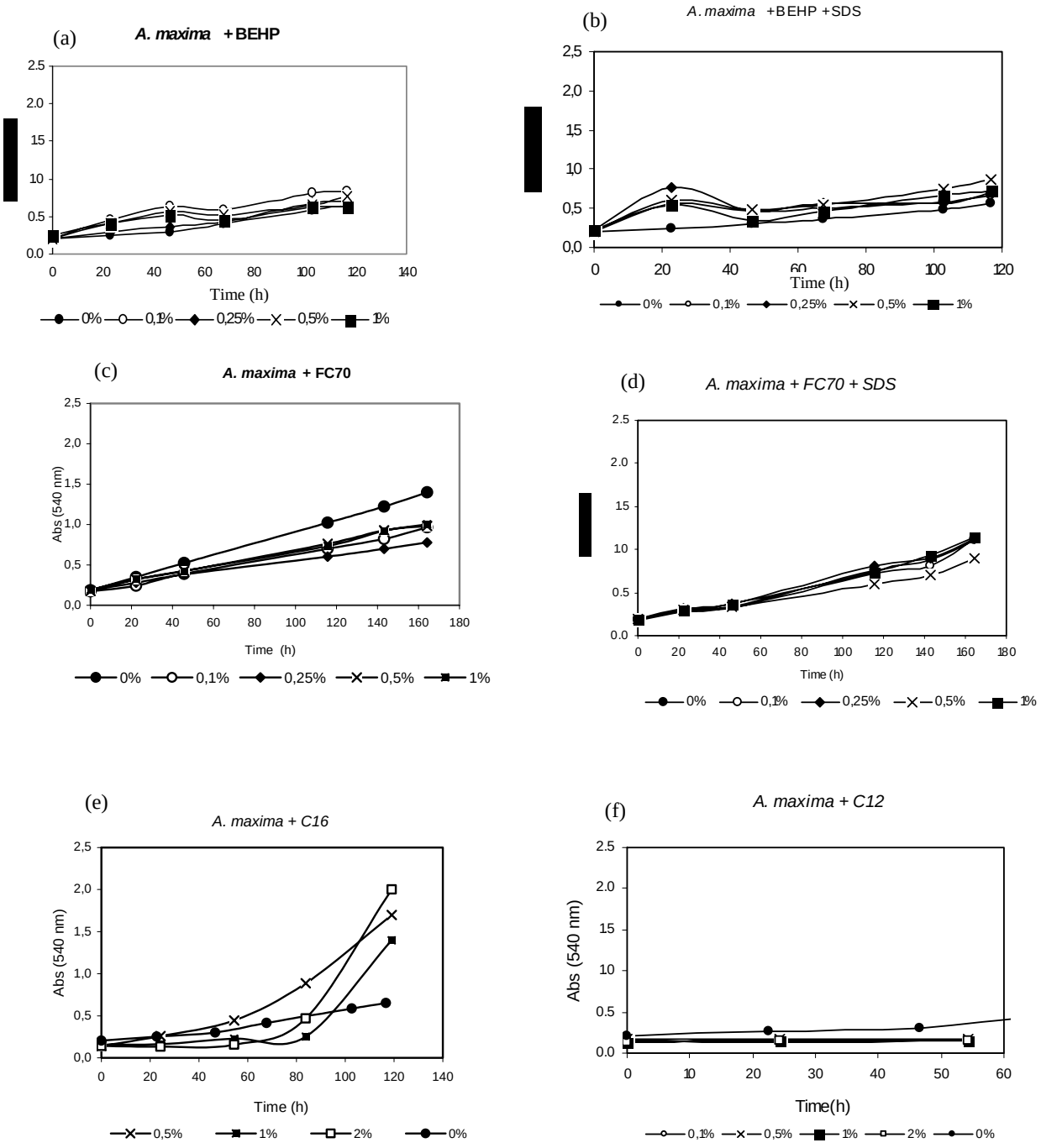

Fig. 1. Absorbance evolution during *Arthrospira (Spirulina) maxima* batch cultures. (a) *Arthrospira (Spirulina) maxima* batch culture with BEHP, at different concentrations; (b) *Arthrospira (Spirulina) maxima* batch culture with BEHP at different concentrations and SDS; (c) *Arthrospira (Spirulina) maxima* batch culture with FC-70, at different concentrations; (d) *Arthrospira (Spirulina) maxima* batch culture with FC-70 at different concentrations and SDS; *Arthrospira (Spirulina) maxima* batch culture with hexadecane, at different concentrations; (e) *Arthrospira (Spirulina) maxima* batch culture with dodecane, at different concentrations

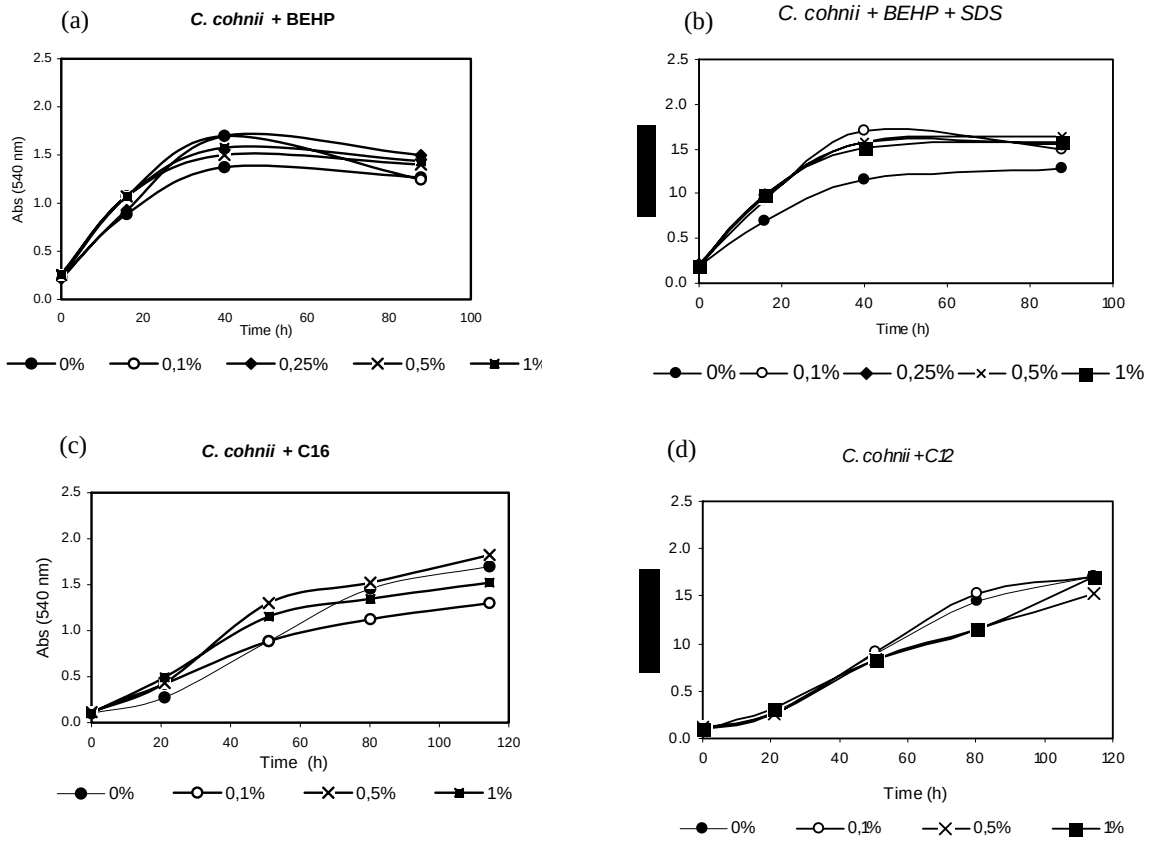

Fig. 2. Absorbance evolution during *Crypthecodinium cohnii* batch cultures. (a) *Crypthecodinium cohnii* batch culture with BEHP, at different concentrations; (b) *Crypthecodinium cohnii* batch culture with BEHP at different concentrations and SDS; (c); *Crypthecodinium cohnii* batch culture with hexadecane, at different concentrations; (e) *Crypthecodinium cohnii* batch culture with dodecane, at different concentrations

The *Arthrospira (Spirulina) maxima* total fatty acids and GLA content also increased when the microorganism grew in the presence of FC-70, attaining their maximums (0.054 g total fatty acids. (g biomass^{-1}) and 0.009 g GLA. (g biomass^{-1})) at the solvent concentration of 0.1 % (v/v) (Table 2). The addition of SDS increased the percentage of GLA at the surfactant concentration of 1% (v/v), reaching 23.6 % of the total fatty acids. From Table 3 it can be seen that the total *C. Cohnii* total fatty acid amount (~19% of biomass) is higher than for *A. maxima* (~4 % biomass). The major fatty acid was DHA, representing about 30% of the total fatty acids, but 14:0 and 16:0 were also present in high proportions (~20% each). The total fatty acids were ~19% of the biomass, whilst the DHA content was 0.061 g GLA (g biomass^{-1}). This result is in accordance with those reported by de Swaaf et al. (1999). The presence of BEHP increased the DHA percentage, attaining about 40% of the total fatty acids at the organic solvent concentration of 0.1% (v/v). The total fatty acids also increased at this solvent concentration, attaining 23.3 % of the biomass. The maximum DHA content was reached at this BEHP concentration, attaining 0.092 GLA.(g biomass^{-1}).

Table 1. *Arthrospira* (*Spirulina*) *maxima* fatty acid composition, total fatty acids and GLA contents in biomass when grown in shake flakes, in the presence of different BEHP concentrations, with or without SDS addition.

		A. maxima									
		Without SDS					With SDS				
% BEHP (v/v)		0	0.1	0.25	0.5	1	0	0.1	0.25	0.5	1
% Fatty acids relatively to total fatty acids	**16:0**	57.6	51.7	31.8	46.8	45.0	57.3	45.1	50.9	52.4	45.6
	16:1	0.0	0.0	4.0	1.6	5.1	2.1	0.0	2.2	7.0	16.2
	18:0	4.8	8.7	1.7	1.6	4.5	2.4	22.5	9.8	2.0	1.9
	18:1ω9	14.8	13.5	11.6	12.2	23.2	3.1	4.8	6.0	0.4	3.9
	18:2ω6	10.6	13.1	36.1	23.6	12.5	17.0	14.6	15.0	17.6	15.3
	18:3ω6 (GLA)	12.2	12.9	14.8	14.1	9.8	18.2	13.1	16.2	20.6	17.1
Total fatty acids in biomass (g g^{-1})		0.041	0.042	0.062	0.029	0.030	0.033	0.036	0.012	0.018	0.008
GLA (18:3ω6) in biomass (g g^{-1})		0.005	0.005	0.009	0.004	0.003	0.006	0.005	0.002	0.004	0.001

Table 2. *Arthrospira* (*Spirulina*) *maxima* fatty acid composition, total fatty acids and GLA contents in biomass when grown in shake flakes, in the presence of different FC-70 concentrations, with or without SDS addition.

		A. maxima								
		Without SDS				With SDS				
% FC-70 (v/v)		0	0.1	0.25	0.5	0	0.1	0.25	0.5	1
% Fatty acids relatively to total fatty acids	**16:0**	45.1	51.0	50.8	50.2	54.3	55.0	43.5	51.0	46.3
	16:1	8.0	5.2	6.8	4.0	5.5	3.2	10.1	7.4	8.5
	18:0	2.9	2.9	2.0	5.1	0.0	0.0	4.1	6.1	1.4
	18:1ω9	7.2	8.0	6.5	9.1	5.3	5.4	5.9	0.6	0.5
	18:2ω6	19.7	15.8	19.3	16.7	17.7	17.9	18.7	17.6	19.7
	18:3ω6 (GLA)	17.0	17.2	14.7	14.8	17.2	18.4	17.7	17.4	23.6
Total fatty acids in biomass (g g^{-1})		0.038	0.054	0.02	0.012	0.033	0.052	0.015	0.008	0.030
GLA (18:3ω6) in biomass (g g^{-1})		0.006	0.009	0.0003	0.002	0.006	0.010	0.003	0.001	0.007

This is remarkable as most marine microalgae rich in polyunsaturated fatty acids contain other fatty acids in such proportions that make the DHA separation and purification a difficult and expensive process. *In C. cohnii* at least three systems appear to be involved in the biosynthesis of the fatty acids (1) the production of

saturated fatty acids, (2) the conversion of saturated to mono unsaturated fatty acids and (3) the production of DHA. In the second step desaturases may be involved which are dependent on oxygen (de Swaaf et al., 2003). Therefore, BEHP might have acted as an oxygen carrier, increasing the oxygen availability in the culture medium. The addition of SDS decreased the total fatty and did not change the DHA content but increased the DHA percentage, reaching 50% of the total fatty acids at the surfactant concentration of 0.1 % (v/v).

Table 3. *Crypthecodinium cohnii* fatty acid composition, total fatty acids and DHA contents in biomass when grown in shake flakes, in the presence of different FC-70 concentrations, with or without SDS addition.

		C. cohnii									
		Without SDS					With SDS				
	% BEHP (v/v)	0	0.1	0.25	0.5	1	0	0.1	0.25	0.5	1
% Fatty acids relatively to toatl fatty acids	**12:0**	6.2	2.6	11.9	5.9	8.3	9.8	3.2	2.9	11.2	10.7
	14:0	19.6	15.5	29.6	21.8	23.3	20.1	16.1	15.8	23.8	23.5
	16:0	19.2	20.0	22.7	22.2	21.3	19.4	23.7	24.0	20.0	19.7
	18:0	2.5	2.5	2.9	2.6	2.7	2.6	2.7	2.8	3.1	2.1
	18:1ω9	9.2	9.6	4.5	4.8	4.6	9.7	4.7	5.0	7.2	5.5
	18:2ω6	10.3	9.5	0.5	-	-	8.1	-	0.3	3.4	2.6
	18:3ω6	0.2	0.3	-	-	-	-	-	0.1	1.7	0.2
	22:5ω3	-	0.5	-	0.8	0.3	-	-	0.4	0.9	-
	22:6ω6 (DHA)	32.9	39.6	27.8	42.3	39.6	30.4	49.6	49.1	29.6	35.8
Total fatty acids in biomass (g g^{-1})		0.186	0.232	0.148	0.144	0.159	0.189	0.114	0.108	0.139	0.145
DHA (22:6ω6) in biomass (g g^{-1})		0.061	0.092	0.041	0.061	0.063	0.057	0.056	0.053	0.041	0.052

Conclusions

These preliminary results suggested that the addition of BEHP to *Arthrospira (Spirulina) maxima* and *Crypthecodinium cohnii* cultures might increase the GLA and DHA productions respectively. Further experiments in CSTR will be done, in order to improve the mass transfer through the addition of the organic solvent carrier. In this case, it is expected to improve both unsaturated fatty acids production.

References

Beach, D.H., Holz, G.G. (1973). Environmental Influences on the Docosahexaenoate Content of the Triacylglycerols and Phosphatidylcholine of a Heterotrophic, Marine Dinoflagellate, *Crythecodinium cohnii*. *Biochim. Biophys. Acta* , 316, 56.

Cohen, Z., Vonshak, A., Richmond, A. (1988). Effect of environmental conditions on fatty acid composition of the red alga *Porphyridium cruentum*: correlation to growth rate. *J. Phycol.*, 24, 328.

de Swaaf, M.E., de Rijk, T.C., van der Meer, P., Eggink, G., Sijtsma, L. (2003). Analysis of docosahexaenoic acid biosynthesis in *Crypthecodinium cohnii* by ^{13}C labelling and desaturase inhibitor experiments. *J. Biotechnol*., 103, 21.

Deziel, E., Comeau, Y., Villemur, R. (1999). Two-Liquid-Phase Bioreactors for Enhanced Degradation of Hydrophobic/toxic Compounds. *Biodegradation*, 10, 219.

Harrington, G.W., Holz, G.G. (1968). The Monoenoic and Docosahexaenoic Fatty Acids of a Heterotrophic Dinoflagelate. *Biochim. Biophys. Acta*, 164, 137.

Hassan, I.T.M., Robison, C.W. (1977). Oxygen Transfer in Mechanically Agitated Aqueous Systems Containing Dispersed Hydrocarbon. *Biotechnol. Bioeng*., 19, 661.

Heipieper, H., Weber, F.J., Sikkema, J., Keweloh, H., Bont, J.A.M. (1994). Mechanisms of Resistance of Whole Cells to Toxic Organic Solvents. TIBTECH, 12, 409.

Kennedy, M.J., Reader, S.L., Davies, R.J. (1993). Fatty Acid Production Characteristics of Fungi with particular Emphasis on Gamma Linolenic Acid Production. *Biotechnol. Bioeng*., 42, 625.

Kyle, D.J., (1996). Production and use of a Single Cell Oil which is Highly Enriched in Docosahexaenoic Acid. Lipid Technol. 8, 107–110.

Kyle, D.J., Sicotte, V.J., Singer, J.J., Reeb, S.E. (1992). Bioproduction of Docosahexaenoic Acid (DHA) by Microalgae. In: Kyle, D.J., Radledge, C. (Eds.), Industrial Applications of Single Cell Oils. *American Oil Chemists' Society, Champaign*, IL, pp. 287.

Lepage, G., Roy, C.C. (1986). Direct transesterification of all classes of lipids in a one-step reaction. *J. Lipid Res.*, 27**,** 114.

Mendes, R.L., Reis, A., Pereira, A.P., Cardoso, M., Palavra, A.F., Coelho, J.P. (2005). Supercritical CO_2 Extraction of gamma-linolenic acid (GLA) from the cyanobacterium *Arthrospira* (*Spirulina*) *maxima. Chem. Eng. J.*, 105, 147.

Molina, E., Fernández, J., Acién, F. G., Chisti, Y.(2001).Tubular photobioreactor design for algal cultures. *J. Biotechnol.*, *92(2*), 113.

Reis, A., Lobo-Fernandes, H.M., Empis, A., Novais, J.M. (1998). Effect of extraction and purification methods on fatty acid composition and gamma-linolenic acid yield and purity from *Arthrospira* (*Spirulina*) *maxima* biomass. In *Marine Microorganisms for Industry.* Y. le gal & A. Muller-Feuga (eds.), Editions IFREMER, ISBN 2-905434-94-5, pp. 34.

Vonshak A. (1986). Laboratory Techniques for the Cultivation of Microalgae. In CRC Handbook of Microalgal Mass Culture, Richmond A., ed., CRC Press, Boca Raton, Florida, pp. 117-145.

Zhao, S., Kuttuva, S.G., Ju, L.K. (1999). Oxygen Transfer Characteristics of Multiple-Phase Dispersions Simulating Water-in-Oil Xanthan Fermentations. .*Bioproc. Eng*., 20, 313.

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